Brain Transplantation of Immortalized Human Neural Stem Cells Promotes Functional Recovery in Mouse Intracerebral Hemorrhage Stroke Model

Hong J. Lee, Kwang S. Kim, Eun J. Kim, Hyun B. Choi, Kwang H. Lee, In H. Park, Yong Ko, Sang W. Jeong and Seung U. Kim

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Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells

D. Eugene Redmond, Jr.*†‡, Kimberly B. Bjugstad§, Yang D. Teng¶, Vaclav Ourednik¶||, Jitka Ourednik¶||, Dustin R. Wakeman**††, Xuejun H. Parsons**, Rodolfo Gonzalez**††, Barbara C. Blanchard§, Seung U. Kim‡‡, Zezong Gu**, Stuart A. Lipton**, Eleni A. Markakis*, Robert H. Roth*§§, John D. Elsworth*§§, John R. Sladek, Jr.§¶¶, Richard L. Sidman‡¶, and Evan Y. Snyder‡¶**

Departments of *Psychiatry, †Neurosurgery, and §§Pharmacology, Yale University School of Medicine, New Haven, CT 06510; §Department of Psychiatry, University of Colorado, Aurora, CO 80045; ¶Departments of Neurology and Neurosurgery, Harvard Medical School, Children's Hospital and Beth Israel Deaconess Medical Center, Division of Spinal Cord Injury Research, Veterans Affairs Boston Healthcare System, Boston, MA 02115; **Burnham Institute for Medical Research, La Jolla, CA 92037; ‡‡Department of Neurology, University of British Columbia, Vancouver, BC, Canada V6T 2B5; and ††Biomedical Sciences and Molecular Pathology Programs, University of California at San Diego, La Jolla, CA 92093

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Stem cells have been widely assumed to be capable of replacing lost or damaged cells in a number of diseases, including Parkinson's disease (PD), in which neurons of the substantia nigra (SN) die and fail to provide the neurotransmitter, dopamine (DA), to the striatum. We report that undifferentiated human neural stem cells (hNSCs) implanted into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated Parkinsonian primates survived, migrated, and had a functional impact as assessed quantitatively by behavioral improvement in this DA-deficit model, in which Parkinsonian signs directly correlate to reduced DA levels. A small number of hNSC progeny differentiated into tyrosine hydroxylase (TH) and/or dopamine transporter (DAT) immunopositive cells, suggesting that the microenvironment within and around the lesioned adult host SN still permits development of a DA phenotype by responsive progenitor cells. A much larger number of hNSC-derived cells that did not express neuronal or DA markers was found arrayed along the persisting nigrostriatal path, juxtaposed with host cells. These hNSCs, which express DA-protective factors, were therefore well positioned to influence host TH+ cells and mediate other homeostatic adjustments, as reflected in a return to baseline endogenous neuronal number-to-size ratios, preservation of extant host nigrostriatal circuitry, and a normalizing effect on α-synuclein aggregation. We propose that multiple modes of reciprocal interaction between exogenous hNSCs and the pathological host milieu underlie the functional improvement observed in this model of PD.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine | dopamine | Parkinson's disease | synuclein | tyrosine hydroxylase

Degeneration of dopamine (DA) neurons in the substantia nigra (SN) and the consequent deficit of DA release in the striatum and other target areas appear to be responsible for the characteristic manifestations of Parkinson's disease (PD). Although substantial improvements result from the systemic administration of the DA precursor L-DOPA or DA agonists, such pharmacological replacement does not address the etiology of the disease, provide a permanent redress of the pathophysiology, or forestall progression of the degenerative process. It does, however, support the idea that DA provided by exogenous replacement cells might be therapeutic, a notion verified in rodents (1–3) and monkeys (4–6), where grafts of fetal DA neurons led to improvements in biochemical and behavioral indices of DA deficiency. However, in graft studies, the improvements in Parkinsonism have been limited and variable (see review in ref. 7). Therefore, we hypothesized that, in addition to DA replenishment, PD treatment should also restore functional equilibrium in the host SN-striatal system. A clinically relevant strategy might be to implant human neural stem cells (hNSCs) and progenitor cells constitutively capable of multiple actions, including neural differentiation and cytokine secretion, and allow them to develop within the PD-affected brains of nonhuman primates to yield cells whose types, numbers, locations, and regulation are determined by the interplay of donor elements and the local host milieu. Outcomes derived from such donor–host interactions may result in a new level of bioequilibrium among the DA-related neurostructures (i.e., homeostasis), which could benefit behavioral recovery. hNSCs, either isolated directly from the developing normal brain (8–11) or derived from embryonic stem cells (12, 13), appear to be well suited for testing implementation of such a hypothesis. As the CNS' most primordial cell, the hNSC has attributes that appear to promote anatomical and functional preservation and/or restoration in neurodegenerative diseases. These properties include the potential for yielding appropriate ratios of cell types that constitute a normal anatomical structure (i.e., both neurons and glia, plus other "chaperone-like" cells) (14–16). In addition, large numbers of hNSCs can be grown in culture as homogeneous, well defined populations. For this study, we used hNSCs directly isolated from a neuroectoderm-derived structure, the telencephalic ventricular zone of normal, early second-trimester human cadavers (8). We selected two identically derived, nonimmortalized hNSC lines (maintained *in*

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Abbreviations: DA, dopamine; DAT, dopamine transporter; hNSC, human neural stem cell; ir, immunoreactive; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; PFS, Parkinson's factor score; GDNF, glial cell line-derived neurotrophic factor; NuMa, nuclear mitotic apparatus.

See Commentary on page 11869.

‖Present address: Laboratory of CNS Development, Regeneration, and Neurotransplantation, Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1250.

¶¶Present address: Department of Biology, California Lutheran University, 60 West Olsen Road, Thousand Oaks, CA 91360.

‡To whom correspondence may be addressed. E-mail: eugene.redmond@yale.edu, richard_sidman@hms.harvard.edu, or esnyder@burnham.org.

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vitro as monolayers in serum-free, mitogen-supplemented medium) for their ability to engraft and migrate *in vivo* (8). One of these cell lines was known to pursue a ventral mesencephalic lineage when presented with appropriate cues *in vitro* (17) and to express a number of markers associated with a mesencephalic cell lineage [supporting information (SI) Fig. 6]. We have reasoned that our hNSC-based comprehensive approach might better alleviate some of the limitations of previous strategies that placed partially differentiated cells, apparently without natural regulatory mechanisms, in ectopic locations such as the striatum (7).

Results

We studied 29 adult male African green monkeys of St. Kitts origin (*Chlorocebus sabaeus*). Four were untreated normal control monkeys, and the remainder were injected systemically with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This neurotoxin causes selective permanent bilateral destruction of mesencephalic DA neurons and their striatal projections, depletion of DA concentrations, and the full signs of Parkinsonism (4, 18, 19). hNSCs were injected stereotaxically into the right SN and bilaterally into caudate nuclei. The approach of implanting undifferentiated (as opposed to precommitted) hNSCs allowed us to investigate whether cues might be present in the host milieu that could permit, or even direct and sustain, an appropriate anatomical and physiological restoration. The animals were studied over periods of ≤8 months and categorized according to MPTP treatment, immunosuppression, numbers of cells injected, and other treatment variables (Groups 1–5; see SI Table 1). To ensure reproducibility, hNSCs were obtained from two separate lines (designated as "H1" and "HFB2050") that were initially derived by the same method: mitogen selection and expansion without immortalization (8). Numerous aliquots of early passaged cells were banked, thawed, and expanded as needed for new studies, hence minimizing cell variability from experiment to experiment over time.

To assess the possible impact of exogenous hNSCs on DA function, we studied a group of severely Parkinsonian monkeys (Group 1). Severity was determined with a well validated and reliable behavioral scoring method consisting of time-sampled, quantitative behaviors and qualitatively rated items that reflect manifestations of Parkinsonism as well as normal behaviors in this primate species. A Parkinson's factor score (PFS), derived from these observations, correlates inversely with postmortem striatal DA concentrations ($r = -0.72$; $n = 18$; $P < 0.01$) (18, 19). Monkeys in this "most severe" category do not spontaneously or significantly improve over periods of ≤1 year (18–20). Furthermore, the PFS in monkeys closely matches the 5-point Hoehn–Yahr scale, which is used clinically to categorize PD patients; the "most severe" category in monkeys corresponds to Stage 5 in humans.

Based on the PFS, eight monkeys that met the "Stage 5–Severely Parkinsonian" criteria were selected for study after their behavioral abnormalities were stable. Stage 5 monkeys show severe difficulty in ambulation, poverty of movement, delayed initiation of movement, lack of responses to food, difficulty eating, periods of "freezing" (remaining motionless for 5 sec), as well as head and limb tremors. The monkeys were randomly assigned to receive hNSC infusions or sham operations. Five hNSC-injected monkeys (10^6 cells × 3 sites per monkey) were compared to three monkeys that received sham surgical injections, with observations starting 4 months before and continuing to 4 months after surgery. The hNSCs, maintained and prepared to optimize engraftment, were injected into the SN and caudate. These severely affected hNSC-injected monkeys improved progressively and were significantly different from controls for the entire posttreatment

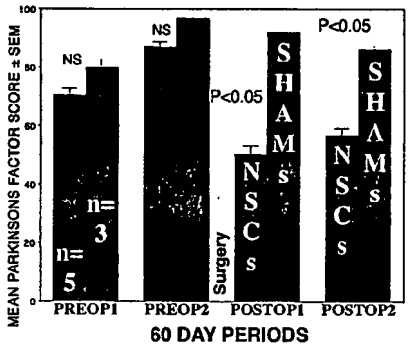

Fig. 1. Behavioral recovery in severely Parkinsonian monkeys after hNSC injections. Severely Parkinsonian monkeys engrafted with hNSC (blue bars) demonstrated a significant decrease in a quantitative PFS, compared to sham-operated monkeys (red bars), which remained severely Parkinsonian. Mean values ± SEM are divided into 60-day periods (PREOP, before injections; POSTOP, after injections). After treatment, the hNSC group improved dramatically and significantly. ANOVA revealed a significant interaction among treatment group (hNSC vs. sham), treatment (before or after), and day of observation ($F = 65.87$, df = 1,1096, $P < 0.0001$). Tests of main effects showed that differences between the treatment groups were not significant before surgery ($F = 1.06$, df = 1,6, P = NS), but became significantly different after ($F = 6.16$, df = 1,6, $P < 0.05$).

period (Fig. 1). These differences were highly significant functionally as well as statistically, and they included "activities of daily living" (such as ability to sit, walk, and self-feed) compared to the sham-injected monkeys, which were unable to do so. Although the hNSC-engrafted monkeys were less improved in the final 60-day period, at the end of the experiment, they remained significantly improved compared to their preimplantation levels and compared to sham-operated monkeys, which remained severely Parkinsonian (Fig. 1). During formal (as well as extended periods of informal) observation of monkeys with "chimeric" human neural cell-bearing brain regions, there were no indications of any qualitative or quantitative behaviors that were not typical of the species, nor were any Parkinsonian dyskinesias noted. The duration and magnitude of functional recovery, compared to the controls, convinced us to terminate the behavioral experiment and begin a more extensive search for biochemical and histological correlates of improvement that might justify longer duration experiments and the investigation of immune and other factors and side effects that might help inform future long-term treatment of human PD.

To understand the basis for this functional recovery, histological sections from brains of these Group 1 monkeys and additional hNSC-injected monkeys were processed to assess the fate of donor and host cells [4 months after hNSC injections, designated as Group 2 (4-month monkeys)]. Another group of MPTP-treated and hNSC-injected monkeys (Group 3) were studied and killed after 7 months (designated as 7-month monkeys). Four monkeys that were sham-operated but not MPTP-treated were controls (Group 4). In Group 1, although hNSCs were injected unilaterally immediately dorsal to the right SN (Fig. 2*A*), we noted that donor-derived cells [identified by BrdU prelabeling (Fig. 2*C*) and β-gal expression (Fig. 2*D*)] were distributed bilaterally throughout the DA pathway, suggesting migration to the contralateral SN (Fig. 2 *B* and *C*) and/or migration from the engrafted ipsilateral caudate. Small numbers of donor-derived cells expressed tyrosine hydroxylase (TH) in the ventral mesencephalic region of Group 1 hNSC-injected monkeys (Fig. 3). Such double-labeled TH+ cells (identified by multiple independent mark-

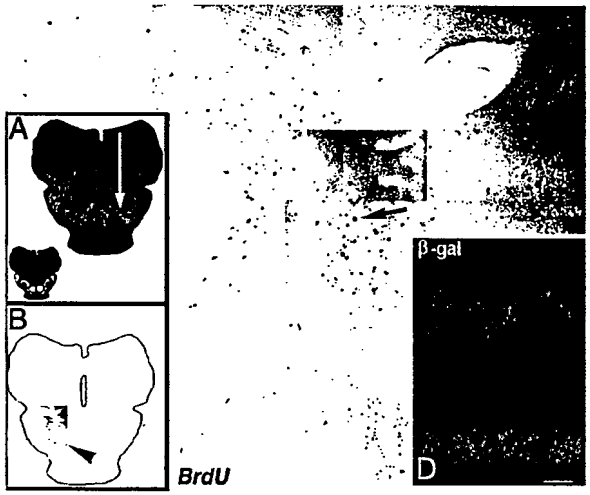

Fig. 2. Survival and migration of engrafted hNSCs associated with functional improvement after transplantation into the nigrostriatal system. (*A*) hNSCs were injected unilaterally, dorsal to the right SN (white arrow) in the monkeys studied behaviorally (Fig. 1, Group 1). Donor-derived cells were mapped (green stars) and detected throughout the area where DA nuclei are located (DA neuron distribution in this region is delineated in orange in the lower left corner, as recorded with a camera lucida). (*B–C*) Donor-derived cells labeled with BrdU and β-gal were also detected on the side contralateral to the implant, as shown by widely distributed BrdU-ir donor-derived hNSCs (black nuclei, black arrow) in the region (arrowhead in *B* and enlarged in *C*). (*D*) β-gal+ cells (green) were present in substantial numbers in the ventral mesencephalon.

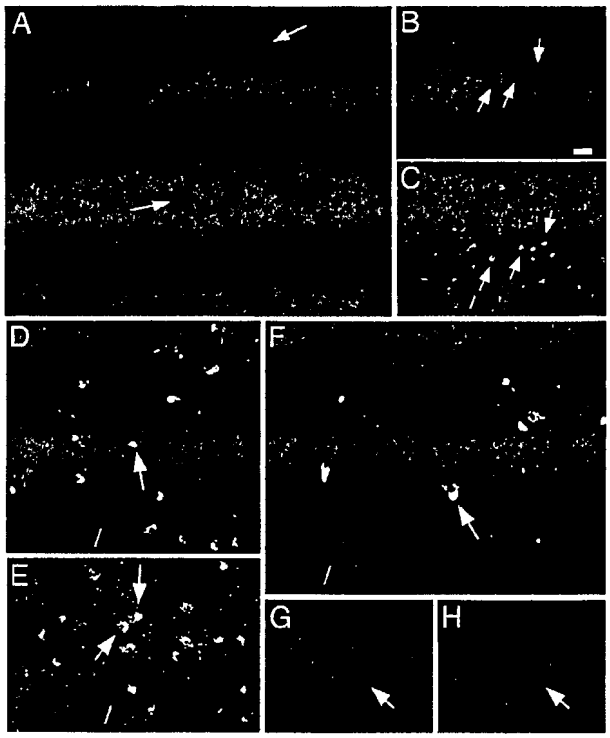

Fig. 3. Additional independent markers identify engraftment and survival of donor-derived hNSCs within structures relevant to Parkinsonism and differentiation of some of them into TH+ cells. Donor-derived cells in the ventral tegmentum are identified by antibodies to the human EGF receptor (hEGFR), nuclear mitotic apparatus (NuMA), or β-gal (arrows). EGFR-ir and NuMA-ir (*A* and *E*) colocalized with *lacZ*-expressing β-gal-ir (*C–E*) donor hNSCs. Furthermore, some donor-derived β-gal-ir cells (*G*) colabeled with TH-ir cells (*H*) in the DA-deficient nigra (*F*) (merged). Confocal microscopic analysis of such cells (with optical dissection and *z*-stacks) is shown in Fig. 4*D* and SI Fig. 7.

ers) were not seen in nonlesioned hNSC-injected adult monkeys, although there was robust survival of hNSCs in all monkeys, whether normal or MPTP-lesioned. (In no monkeys were neoplasms, tumors, deformation, or overgrowth noted.)

To confirm the presence and numbers of hNSC-derived neurons expressing markers consistent with a DA phenotype, we injected hNSCs into six additional MPTP-lesioned monkeys and performed additional histological studies on them after >7 months (SI Table 1, Group 5). Although in the earlier studies no differences were noted between cyclosporine- and noncyclosporine-treated animals, azathiaprine and prednisolone were added to cyclosporine in this later group of monkeys to increase immunosuppression. These animals showed extensive survival of hNSCs, yielding a variety of neural cell types, including significant numbers of TH+ and DAT+ expressing cells in the disabled SN (Fig. 4 and SI Fig. 7). Although such cells constituted ≤1% of donor-derived cells in the SN, they represented 4–7% of the total TH+ cellular population in that region. Further, because of migration of the unilaterally injected hNSCs to the contralateral equally impaired SN, the percentage of TH+ cells that were donor-derived was not significantly different between the two sides [implanted, 6.74 ± 1.75% vs. unimplanted, 5.99 ± 1.74%; $F(1, 8) = 0.15$, P = NS]. Accordingly, the actual concentrations of DA measured biochemically in punches from these regions were also statistically not different [$t(3) = 0.087$, P value not significant]. The number of BrdU+ cells that were also TH+ was not significantly different from those that were also DAT+ [TH+, 6.37 ± 1.23% vs. DAT+, 4.69 ± 1.03%; $F(1, 8) = 0.70$, P value not significant]. (Because not all hNSCs become prelabeled *ex vivo* with BrdU, a larger number of TH+ and/or DAT+ cells in the SN may have been derived from donor hNSCs.) There was no significant difference between the total number of counted BrdU-labeled cells between the implanted and unimplanted sides (5,931 ± 312 vs. 4,672 ± 988, respectively) [$t(3) = 1.86$, P value not significant].

Besides bilateral distribution of hNSCs in the SN after a unilateral injection, the hNSCs and their progeny appeared to migrate from the depleted striatum toward the SN along the nigrostriatal pathway (Fig. 5 *A* and *B*). Most donor-derived cells were found between SN and the striatum and ventral to the SN. Donor hNSC-derived TH− cells were closely associated with host-derived cell bodies and TH+ fibers in the SN and nigrostriatal pathway (Fig. 5*B*). Indeed the close physical association suggested that stimulus–response intercellular relationships might be in process between donor-derived nonneuronal cells and host DA neurons and their fibers (Fig. 5*B*). This nonrandom distribution pattern was reminiscent of one of the proposed routes followed by progenitors during embryonic emergence of the nigrostriatal functional unit and might suggest that this pathway can still be used by progenitors, with possible behavioral consequences in the adult primate brain with PD pathology. No cells in the striatum of any of the monkeys were double-labeled for TH+ and β-gal, nuclear mitotic apparatus (NuMA), or other human-specific markers studied. (Because of the extensive migration of hNSCs and their progeny, it was not feasible to count the proportion of implanted cells that survived. A more detailed study of cell migration in these monkeys is in progress.)

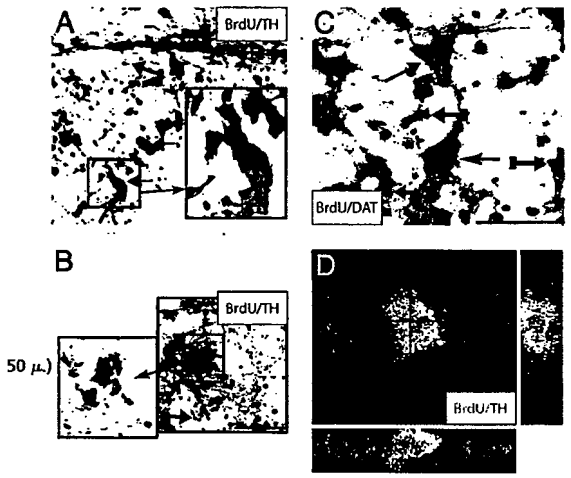

Fig. 4. Some hNSCs transplanted into the SN of MPTP-lesioned monkeys showed key markers of DA neurons. (*A*) A black BrdU-ir nucleus indicates a donor-derived cell, with a small proportion also containing brown cytoplasmic TH-ir (6.75 ± 1.28% of all TH+ cells, red arrows). (*Inset*) Magnification of blocked cells. (*B*) Most BrdU+ cells are not TH+ (black arrows). Compared with host BrdU-negative TH+ cells in *B* (blue arrows), cells in *A* are most likely donor-derived. (*Inset*) Magnification of blocked host BrdU-negative TH+ cells. (*C*) Some donor-derived BrdU+ cells in this region were also immunoreactive for the DAT (red arrow with tail); 3.91 ± 1.04% of DAT+ cells were also labeled with BrdU. A DAT+ neuron with a nucleus void of BrdU (blue arrow), presumably an endogenous host cell, is seen above the hNSC-derived neuron (red arrow), as well as many DAT-negative BrdU+ (black nucleus) hNSCs (black arrows with tails) juxtaposed to DAT+ fibers. (*D*) Double-label immunofluorescence of an hNSC cell expressing TH viewed by confocal microscopy with *z*-stacks; a BrdU+ nucleus (red) is surrounded by a TH+ cytoplasm (green). Red and blue lines indicate corresponding points in the orthogonal planes, confirming localization of the label within the pictured cell after the summation of serial optical sections. See also SI Fig. 7. (Scale bars: *B*, 100 μm; *C*, 50 μm.)

We also noted significant increases in the size of host TH+ neurons in the SN by 7 months after hNSC injections (Fig. 5*C* and SI Fig. 8 *A* and *B*), associated with the presence of donor hNSC that were not TH+ or DAT+. Thus, hNSCs appeared to exert homeostatic effects on host circuitry, increasing the size of abnormally small endogenous TH+ neurons of the SN toward normal values. MPTP-induced changes in the size and number of TH+ host cells in the striatum were also normalized after hNSC injections (SI Fig. 9). Although the molecules mediating the impact of hNSCs on host DA systems are unknown, some BrdU+ cells expressed a marker associated with an astrocytic lineage (Fig. 5*G*) and expressed glial cell line-derived neurotrophic factor (GDNF) (Fig. 5*H*), a growth factor known to augment and/or protect DA systems (21–24). Also, increased aggregation of α-synuclein has been reported after MPTP treatment in rodents (25) and primates (26). We found by immunohistochemical analysis of the nigrostriatal system in eight animals that α-synuclein aggregation was present in >80% of cells in monkeys that were MPTP-exposed only, but aggregation was found in <20% of cells after hNSC implantation had followed MPTP exposure (Fig. 5*I* and SI Fig. 10). No aggregates were seen in non-MPTP-lesioned monkeys regardless of whether they were transplanted with hNSCs. In summary, hNSC implantation appeared to return a number of abnormalities after MPTP lesioning to the parameters seen in normal animals.

Discussion

Our studies demonstrate that the MPTP-lesioned adult monkey brain retains intrinsic microenvironmental signals that may direct differentiation of an uncommitted human stem cell toward a DA phenotype and suggest that hNSCs have the capacity to respond to a DA deficiency (27) even without preinduction by factors or transgenes. However, the predominant functional action of hNSCs in the presence of damage to DA systems was most likely one of promoting homeostatic adjustment of host nigral DA neurons and their nigrostriatal projections. Some of the hNSCs, particularly those juxtaposed to host cells and fibers along the nigrostriatal trajectory, pursued an astrocytic lineage, which included expression of the neurotrophic factor GDNF. This observation is consistent with previous findings that epigenetic signals promoting the differentiation of Nurr1-expressing precursors may emanate from neighboring astrocytes (28). In fact, the role of the astrocyte in directing neurogenesis (15), mediating rescue (14), and potentiating the function of other neural cells is becoming increasingly appreciated (29). In particular, GDNF (one of possibly several natural products of the astrocytic progeny of these hNSCs) shows developmental, trophic, and protective support of DA neurons and promotes effective processing and release of DA (21–23). The normalization of α-synuclein aggregation in the presence of injected hNSCs in this study illustrates another potentially beneficial effect of hNSCs, but is probably independent of GDNF (30).

Most prior studies have focused on the concept that the host environment, as it changes during the course of development and aging, or after injury or cell degeneration, influences the transplanted stem cell, as exemplified here by the homeostatic emergence of some donor-derived TH+ and DAT+ cells. Based on past reports that small reversals of DA depletion can underlie large functional improvements, even a small elevation in DA might be behaviorally relevant, whether from stem cell differentiation into DA neurons or from preservation and even augmentation of host DA pathways via hNSC-derived trophic/neuroprotective effects. This study also reveals that improvement in function might result from significant reversal of abnormalities in sizes and distributions of endogenous TH+ cells in the nigrostriatal system as well as an interesting normalization of α-synuclein aggregation. These effects are consistent with other studies that have shown NSCs yielding multiple interacting cell types, not only key effector neurons but also undifferentiated progenitor cells that mediate neuroprotection and neuroplasticity (31) and glia that nurture, detoxify, myelinate, or direct the differentiation of neurons (32, 33). NSCs have also been suggested to restore equipoise to a disequilibrated milieu by fueling cell turnover (8, 34) and regulating gene expression and signaling pathways (35, 36). We believe that our data suggest, therefore, that the Parkinsonian primate CNS may benefit from such homeostatic effects, including (*i*) replacement of degenerating DA neurons by differentiated human stem cells, and (*ii*) the trophic, protective, and guidance effects of nonneuronal stem cell-derived progeny. These latter actions may manifest themselves by promoting recovery through the variety of effects described here, as well as by others that remain to be elucidated by additional experiments and controls. Although long-term studies of these effects and potential side effects, such as dyskinesia (although not observed in the present study) and possible immunorejection of exogenous stem cells, are needed before attempting clinical application, this report provides evidence that permissive signals are present in the milieu, and that stem cells respond with multiple homeostatic actions to restore functionality to an adult primate brain that presents with severe Parkinsonian pathology.

Materials and Methods

Source and Maintenance of hNSCs. Cells were obtained from stable, self-maintaining populations of hNSCs dissected from the ventricular germinal zone of a 13-week-old human fetal cadaver (8, 37) and maintained in neurobasal (Gibco/Invitrogen, Grand Island, NY) medium supplemented with N2

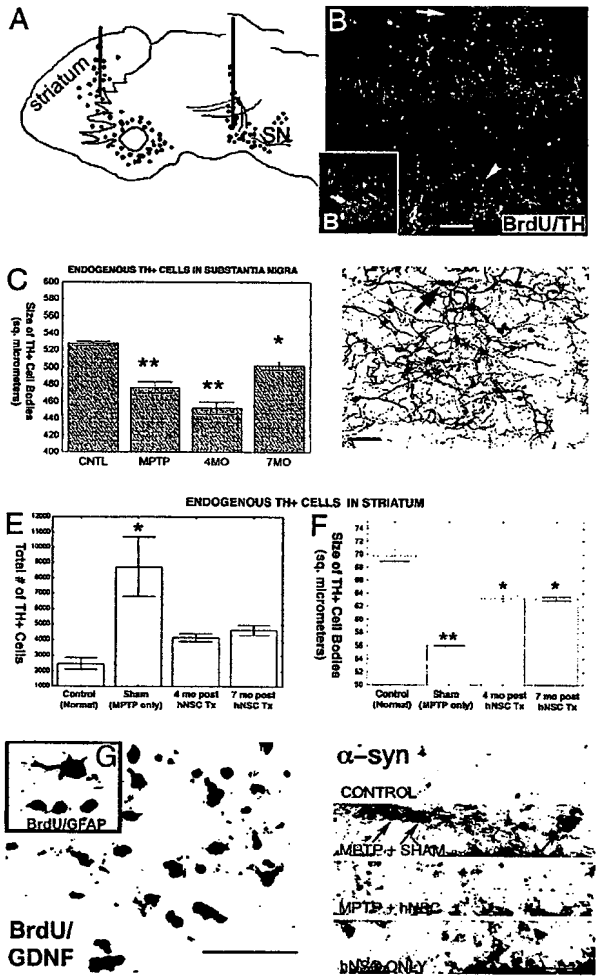

Fig. 5. hNSC engraftment is associated with multiple influences on the host DA nigrostriatal system that might contribute to the observed functional improvement. Migration of hNSCs, normalization of pathological numbers and sizes of host TH+ cells, and effects on α-synuclein aggregation are shown, with evidence of secretion of a growth factor known to preserve fibers in the host nigrostriatal system. (*A*) Four and 7 months after hNSCs were placed in the SN and caudate, the majority of donor-derived BrdU+ cells had migrated to the nigrostriatal pathway as illustrated in a composite of serially sampled sections from an entire brain. Red dots represent an approximate density and locations where the majority of BrdU+ cells were found. Green dots and lines indicate host TH+ cells and their fibers. Blue lines indicate the locations where hNSCs had been implanted. (*B*) Many nonneuronal hNSC-derived cells (BrdU+ in red, marked by white arrow) were found in the SN and closely associated with host TH+ cell bodies and their neurites (green, marked by white arrowhead). (*Inset*) Robust, healthy host DA neuronal soma with extensive processes (see also SI Fig. 8 *A* and *B*). (*C*) In the SN, MPTP reduced the size of host TH+ cells, which were then significantly increased 7 months after hNSC injections, compared to sham-operated MPTP-lesioned monkeys [ANOVA post hoc group differences; *, smaller than corresponding control group only; **, smaller than all other treatment groups ($P < 0.05$)]. (*D*) Endogenous TH+ cells are also found in small numbers in the primate striatum. The arrow points to the most prominent type of striatal TH+ neuron, which is small and bipolar (see SI Fig. 9 *A–D*). (*E* and *F*) Their size-to-number ratios become disordered after MPTP lesioning. After MPTP lesioning, striatal TH+ neurons increase in number (*E*) and decrease in size (*F*), a compensatory but abnormal change. They do not restore DA function and, in fact, are at their peak in animals that show the greatest signs of DA deficiency. In monkeys receiving hNSC implants, the aberrant size-to-number relationships of striatal DA neurons return to near normal control parameters (see SI Fig. 9). (*G* and *H*) Some hNSCs (BrdU+ cells, black nuclei) along the nigrostriatal pathway were also immunoperoxidase-positive (brown cytoplasm) for glial fibrillary acidic protein (*G*) and GDNF (*H*), suggesting that they had differentiated into astrocytes spontaneously and constitutively produced this trophic factor as a potential mechanism for hNSCs' effects on host neurons. (*I*) hNSCs transplanted into MPTP-lesioned monkeys appeared to diminish the α-synuclein-ir aggregation pattern (arrows) in the host striatum, approximating a more normal profile (as seen in nonlesioned monkeys with and without hNSCs). (Scale bars: *B*, 100 μm; *D*, 20 μm; *H* and *I*, 50 μm.)

or B-27 (Gibco) plus bFGF (20 ng/ml) (Chemicon International, Temecula, CA), heparin (8 mcg/ml), and LIF (5 ng/ml) (Chemicon International). Both adherent cells and floating clusters were chemically dissociated with Accutase (Chemicon International), triturated, and passaged every 3 to 10 days. hNSC lines (8, 37) were propagated with mitogens alone. More details are provided in *SI Materials and Methods*.

MPTP Lesioning of Monkeys and Treatment Groups. Five groups of monkeys were studied with or without injections with MPTP·HCl (RBI/Sigma–Aldrich, Natick, MA). Seventeen monkeys received cumulative doses of 2.25 mg/kg over a 5-day period to induce degeneration of the nigrostriatal pathway, and six monkeys received 1.5 mg/kg aimed to produce DA depletion but without functional impairments. Seven monkeys were sham-injected, and 20 received hNSCs injections. Four monkeys, which were not treated with MPTP, were studied as controls (see SI Table 1 for individual details of cell numbers, immunosuppression, cell types, and numbers). The animal experiments were approved by the relevant institutional animal care and use committees of the collaborating institutions.

Behavioral Scoring and Statistical Analysis. Blinded observers scored the MPTP-treated monkeys by using a published and validated quantitative time-sampling method (4, 18) two periods per day, 5 days per week, a regime that has been shown empirically to sample Parkinsonian behaviors efficiently and accurately. Statistical analysis of behavioral changes used a multifactor ANOVA of the daily PFS of each monkey. All 1,304 individual observations were analyzed in 60-day blocks from 120 days before to 120 days after hNSC implantation, when monkeys were killed; >95% concordance was recorded among five blinded observers for all behaviors tested.

Preparation and Transplantation of hNSCs. hNSCs were injected into the right SN and the right and left caudate nuclei by using stereotaxic procedures. Donor-derived cells were identified by multiple independent techniques. Dissociated hNSCs were preincubated *ex vivo* with BrdU for 48 to 72 h *in vitro* before transplant. Some hNSCs were subcloned to stably express the *lacZ* transgene and produce β-gal. Control experiments confirmed that donor cell-specific markers were never transferred to host cells after cell destruction; donor cells never produced recombinant replication-competent helper virus. hNSCs were dissociated and passaged 24 to 48 h before transplantation to help synchronize and make as uniform as possible their state of differentiation and stage in the cell cycle (8, 37). The number of hNSCs injected was 1,000,000 cells in most animals, although it ranged from 1 to 8.75 E + 06 in Group 5. Additional monkeys were MPTP-treated only and received injections of vehicle or needle passage alone ("sham-transplanted") (see SI Table 1).

Histological Analyses. Donor-derived and host cells were distinguished in postmortem fixed tissue by immunocytochemistry with antibodies against multiple independent markers, including BrdU, β-gal, and human-specific epitopes (8, 37, 38). Unbiased stereology was used for counting labeled cells. The number and size of TH+

immunoreactive (ir) cells in the caudate and SN were quantified, and BrdU+, TH+, and DAT+ cells were calculated as a percentage of total cells of each type. Immunocytochemistry for α-synuclein was performed with standard methods and primary antibody (Cell Signaling Technology, Danvers, MA) at 1:500 dilution and second antibody (Jackson ImmunoResearch, West Grove, PA) at 1:200.

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1. Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ (1979) *Science* 204:643–653.
2. Björklund A, Stenevi U (1979) *Brain Res* 177:555–560.
3. Brundin P, Strecker RE, Widner H, Clarke DJ, Nilsson OG, Åstedt B, Lindvall O (1988) *Exp Brain Res* 70:192–208.
4. Redmond DE, Jr, Sladek JR, Jr, Roth RH, Collier TJ, Elsworth JD, Deutch AY, Haber S (1986) *Lancet* 1:1125–1127.
5. Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Collier TJ, Redmond DE, Jr (1991) *Exp Brain Res* 85:335–348.
6. Bankiewicz K, Mandel RJ, Sofroniew MV (1993) *Experimental Neurology* 124:140–149.
7. Redmond DE, Jr (2002) *Neuroscientist* 8:457–488.
8. Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, *et al.* (1998) *Nat Biotechnol* 16:1033–1039.
9. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL (2000) *Proc Natl Acad Sci USA* 97:14720–14725.
10. Vescovi A, Snyder E (1999) *Brain Pathol* 9:569–598.
11. Villa A, Snyder EY, Vescovi A, Martinez-Serrano A (2000) *Exp Neurol* 161:67–84.
12. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001) *Nat Biotechnol* 19:1129–1133.
13. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, *et al.* (2002) *Nature* 418:50–56.
14. Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002) *Nat Biotechnol* 20:1103–1110.
15. Song HJ, Stevens CF, Gage FH (2002) *Nat Neurosci* 5:438–445.
16. McKay IJ, Lewis J, Lumsden A (1997) *Eur J Neurosci* 9:1499–1506.
17. Daadi MM, Weiss S (1999) *J Neurosci* 19:4484–4497.
18. Taylor JR, Elsworth JD, Sladek JR, Jr, Roth RH, Redmond DE, Jr (1994) in *Toxin-Induced Models of Neurological Disorders*, eds Woodruff ML, Nonneman AJ (Plenum, New York), pp 139–174.
19. Elsworth JD, Taylor JR, Sladek JR, Jr, Collier TJ, Redmond DE, Jr, Roth RH (2000) *Neuroscience* 95:399–408.
20. Taylor JR, Elsworth JD, Roth RH, Sladek JR, Jr, Redmond DE, Jr (1997) *Neuroscience* 81:745–755.
21. Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, Simmerman L, Russell D, Martin D, Lapchak PA, Collins F, *et al.* (1996) *Nature* 380:252–255.
22. Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC (1997) *Science* 275:838–841.
23. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, *et al.* (2000) *Science* 290:767–773.
24. Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005) *Ann Neurol* 57:298–302.
25. Vila M, Vukosavic S, Jackson-Lewis V, Neystat M, Jakowec M, Przedborski S (2000) *J Neurochem* 74:721–729.
26. Kowall NW, Hantraye P, Brouillet E, Beal MF, McKee AC, Ferrante RJ (2000) *NeuroReport* 11:211–213.
27. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, *et al.* (2002) *Proc Natl Acad Sci USA* 99:2344–2349.
28. Wagner J, Akerud P, Castro D, Holm P, Snyder E, Perlmann N, Arenas E (1999) *Nature Biotechnol* 17:653–659.
29. Talbott JF, Loy DN, Liu Y, Qiu MS, Bunge MB, Rao MS, Whittemore SR (2005) *Exp Neurol* 192:11–24.
30. Lo Bianco C, Deglon N, Pralong W, Aebischer P (2004) *Neurobiol Dis* 17:283–289.
31. Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002) *Proc Natl Acad Sci USA* 99:3024–3029.
32. Song H, Stevens CF, Gage FH (2002) *Nature* 417:39–44.
33. Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) *Cell* 120:421–433.
34. Rosario CM, Yandava BD, Kosaras B, Zurakowski D, Sidman RL, Snyder EY (1997) *Development (Cambridge, UK)* 124:4213–4224.
35. Mehler MF, Gokhan S (2001) *Prog Neurobiol* 63:337–363.
36. Li J, Imitola J, Snyder EY, Sidman RL (2006) *J Neurosci* 26:7839–7848.
37. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, *et al.* (2004) *Proc Natl Acad Sci USA* 101:18117–18122.
38. Ourednik V, Ourednik J, Flax JD, Zawada WM, Hutt C, Yang C, Park KI, Kim SU, Sidman RL, Freed CR, *et al.* (2001) *Science* 293:1820–1824.